Vol. 93, No. 2, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 28, 1980 Pages 516-520

IMPAIRED DEOXYURIDINE UTILIZATION IN THE B_{12}-INACTIVATED RAT AND ITS CORRECTION BY FOLATE ANALOGUES

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Received February 4,1980

SUMMARY

Rats were kept in an atmosphere of 50% N_2O in order to inactivate cob(I)alamin. There was an impaired utilization of deoxyuridine for DNA synthesis by marrow cells from these animals. The defect was not improved by the addition of hydroxocobalamin. Formylated tetrahydrofolates corrected the defect but tetrahydrofolate and 5-methyltetrahydrofolate produced either little or no improvement. Thus formyltetrahydrofolates overcome both the impairment of folate polyglutamate synthesis [11] and the impaired deoxyuridine utilization which follows N_2O-induced oxidation of the B_{12} coenzyme.

Exposure to the anaesthetic gas nitrous oxide (N_2O) leads to rapid oxidation of vitamin B_{12} from the active reduced cob(I)alamin form to the inactive oxidized cob(III)alamin form. This occurs both *in vitro* [1,2] and *in vivo* in man [3,4,5] and animals [6,7]. The activity of B_{12}-dependent methionine synthetase in rat liver declines after 30 m exposure to N_2O and is undetectable after 360 m [7]. In man megaloblastic haemopoiesis is produced by N_2O inhalation [3,5], but haemopoiesis in animals remains normoblastic. One of the features of a megaloblastic marrow is impaired methylation of deoxyuridine to form deoxythymidine [8] and this step is tested in the deoxyuridine suppression test [9]. Although the marrows of animals exposed to N_2O remain normoblastic, an impairment of deoxyuridine utilization appears within an hour of breathing 50% N_2O [7,10].

We have shown that livers of N_2O-treated rats fail to synthesize folate polyglutamate from either H_4PteGlu or 5-CH_3-H_4PteGlu but do so normally

Abbreviations: nitrous oxide, N_2O; tetrahydropteroylglutamic acid, H_4PteGlu; 5-methyltetrahydropteroylglutamic acid, 5-CH_3-H_4PteGlu; 10-formyltetrahydropteroylglutamic acid, 10-CHO-H_4PteGlu; 5-formyltetrahydropteroyglutamic acid, 5-CHO-H_4PteGlu; 5,10-methylenetetrahydropteroylglutamic acid, 5,10-CH_2-H_4PteGlu; pteroylglutamic acid, PteGlu; hydroxocobalamin, OH-Cbl; deoxyuridine, dU.

0006-291X/80/060516-05$01.00/0

from 5-CHO, 10-CHO and 5,10-CH=H_4PteGlu [11]. The purpose of this study was to determine whether the abnormal deoxyuridine utilization by marrow cells from the N_2O-treated rat was improved by those folate analogues that were effective in restoring normal folate polyglutamate synthesis.

MATERIALS AND METHODS

Animals: Male, Sprague-Dawley, 80-120 g rats were placed in a chamber in which a mixture of N_2O (50%)/oxygen (50%) was passed and CO_2 and humidity controlled. After 3 hours the animals were killed by exsanguination preceded by an injection of sodium pentobarbitone, long bones removed, split with a scalpel and marrow washed into 5 ml cold Hanks balanced salt solution (BSS) (pH 7.2-7.4) containing 0.5 ml preservative-free heparin (1000 iu/ml). Control animals were left in air.

Marrow: The marrow collected in Hanks solution was passed through 21 and 25 gauge needles to break up clumps and washed in cold Hanks BSS. The cells were then resuspended in a known volume of Hanks BSS containing 10% rat serum. A cell count was performed and volume adjusted so that there were between 1.5 x 10^6 to 7 x 10^6 cells/ml.

Solutions: The following were added in the deoxyuridine (dU) suppression test in 10 µl volumes both to the tubes to contain dU and to the matched controls from which only dU was omitted: hydroxocobalamin 1 mg/ml, dl 5-CHO-H_4PteGlu (Lederle) 3 mg/ml, PteGlu 1.5 mg/ml, dl 5-CH_3-H_4PteGlu (Sigma) 3 mg/ml in 0.1 M potassium phosphate buffer pH 7.0 to which was added 10 mM 2-mercaptoethanol, dl H_4PteGlu (Sigma) 3 mg/ml in 1% potassium ascorbate pH 6.5 stored under nitrogen, 10-CHO-H_4PteGlu 3 mg/ml prepared from 5-CHO-H_4PteGlu (Sigma) by the method of Rabinowitz [12], in 1% potassium ascorbate pH 7 and kept under nitrogen.

Deoxyuridine suppression test [8,9,13]. In this test a marrow cell suspension is incubated with deoxyuridine. This is normally converted into thymidine which in turn is incorporated into DNA. At a second stage [^{3}H]thymidine is added so that any requirements not met by synthesis from dU is met by utilization of [^{3}H]thymidine. A 100% value is obtained by incubating marrow with [^{3}H]thymidine directly without preceding dU. The result is expressed as

$$\frac{[^3\text{H}]\text{thymidine uptake after dU} \times 100}{[^3\text{H}]\text{thymidine uptake alone}}$$

Normal rat (or human) marrow meets more than 90% of its thymidine requirements from dU so that less than 10% of [^{3}H]thymidine is used.

Tests and controls were set up in duplicate. One ml marrow suspension was added to each tube followed by 10 µl of the additive (cobalamin and/or a folate analogue) and the tubes incubated at 37°C for 15 m. Deoxyuridine (Sigma) 0.33 umol in 10 µl saline was added to one set of tubes and incubation at 37°C continued for 15 m. [^{3}H]thymidine (1µCi/0.2 nmol) (Amersham) in 100 µl saline was added to all tubes and incubated for 60 m. The cells were washed twice in cold phosphate buffered saline, 2 ml cold 0.5M perchloric acid added to the cell pellet and left in ice for 10 m. The supernatant was decanted and DNA extracted in 0.5 ml 0.5M perchloric acid for 20 m at 80°C.

[^{3}H]thymidine was measured in 100 µl of the clear extract by liquid scintillation, appropriate correction being made for quenching.

RESULTS

These are shown in the Table and figure. The mean dU result in marrows from 40 control rats was 7.3%, that is 92.7% of thymidine requirements had been met by methylation of deoxyuridine and only 7.3% from preformed [^{3}H]thymidine. The 100% value in each set are the counts in the tubes containing [^{3}H]thymidine in the absence of deoxyuridine but in the presence of the appropriate additive which can of itself influence the amount of thymidine incorporated into DNA. There was no significant change in control marrows with any of the additives other than the lower mean value on addition of 10-CHO-H_4PteGlu this being just significant at the 5% level.

After 3 hours exposure to 50% N_2O the dU suppression was abnormal the mean being 15.7%. Unlike the results reported in man after N_2O exposure

Table: Results of the deoxyuridine suppression test with marrow cells in control and N_2O-treated rats and the effect of addition of hydroxocobalamin and/or folate analogues. Results are expressed as: $\frac{\text{counts with deoxyuridine + [}^3\text{H]thymidine}}{\text{counts with [}^3\text{H]thymidine}} \times 100$

Additive	Controls			N_2O-treated		
	No.	Mean	S.D.	No.	Mean	S.D.
nil	40	7.3	1.57	21	15.7	2.46
OH-Cbl	8	6.8	1.62	8	15.0	2.31
PteGlu	4	6.7	1.17	4	14.7	2.13
H_4PteGlu	8	6.5	0.89	8	12.8	2.02
5-CH_3-H_4PteGlu	8	6.3	1.55	8	14.5	2.86
5-CHO-H_4PteGlu	10	7.5	1.66	10	9.2	1.44
10-CHO-H_4PteGlu	8	6.2	1.22	8	9.4	1.89
OH-Cbl + PteGlu	4	7.2	1.89	4	13.8	1.55
OH-Cbl + H_4PteGlu	8	7.4	1.33	8	13.5	2.90
OH-Cbl + 5-CH_3-H_4PteGlu	8	6.6	1.57	8	14.1	1.56
OH-Cbl + 5-CHO-H_4PteGlu	8	7.5	1.80	8	8.9	1.83
OH-Cbl + 10-CHO-H_4PteGlu	8	7.4	1.26	8	12.6	2.58

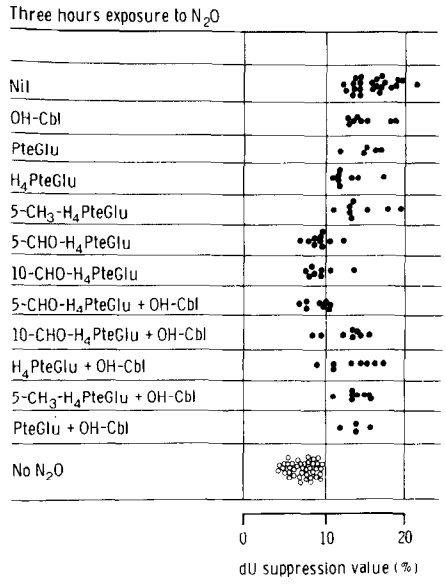

Figure. Results of the deoxyuridine suppression test with marrow cells from N_2O-treated rats and the effect of hydroxocobalamin and/or folate analogues. Results with marrow from rats breathing air are also shown. All results are expressed as:

$$\frac{\text{Counts with deoxyuridine + [}^3\text{H]thymidine}}{\text{Counts with [}^3\text{H]thymidine}} \times 100$$

[5] there was no improvement on the addition of B_{12}. There was no significant improvement in dU utilization on the addition of PteGlu and 5-CH_3-H_4PteGlu. The mean value fell to 12.8% on the addition of H_4PteGlu. The most significant improvement in utilization of dU occurred with the addition of 5-CHO and 10-CHO-H_4PteGlu the mean values being 9.2 and 9.4% respectively. Most of the values fell into the normal range (figure). There was no further improvement when B_{12} was added with a folate analogue.

DISCUSSION

The results follow the pattern described in relation to folate-polyglutamate synthesis in the N_2O-treated rat. The abnormal utilization of dU is corrected most effectively by formyltetrahydrofolates, much less well by H_4PteGlu and not at all by PteGlu or 5-CH_3-H_4PteGlu.

Surprisingly, B_{12} was ineffective. Others have reported some improvement [5] with B_{12} particularly when an interval was allowed

between withdrawal of N_2O and the test [14], perhaps when regeneration of the apoenzyme had occurred. The relative ineffectiveness of H_4PteGlu also occurs with pernicious anaemia marrows (to be published) and with other observations in N_2O-treated animals [11,15] suggest that trapping of H_4PteGlu (the methylfolate trap hypothesis) is not the correct explanation for its ineffectiveness in overcoming the B_{12} block.

Our data indicate the formyltetrahydrofolates are the most effective analogues in overcoming the N_2O-induced block in B_{12} metabolism and hence that B_{12} has a role in formylation of folate. The N_2O-treated rat shows a defect in the serine-glycine reaction (unpublished observations) and this failure to supply formate may lead to lack of CHO-H_4PteGlu which appears to be the substrate for folatepolyglutamate synthesis [11]. Failure to provide 5,10-CH_2-H_4PteGlu may also account for the impaired dU methylation. The more precise role of B_{12} however, remains to be determined.

References

1. Banks, R.G.S., Hendersen, R.J. and Pratt, J.M. (1968) J.Chem.Soc. (A) 2886-2889.
2. Blackburn, R., Kyaw, M. and Swallow, A.J. (1977) J.Chem.Soc.Farad. Trans. 73, 250-255.
3. Lassen, H.C.A., Henricksen, E., Neukirch, F. and Kristensen, H.S. (1956) Lancet, i, 527-530.
4. Wilson, P., Martin, F.I.R. and Last, P.M. (1956) Lancet, ii, 442-443.
5. Amess, J.A.L., Burman, J.F., Rees, G.M., Nancekievill, D.G. and Mollin, D.L. (1978) Lancet, ii, 339-342.
6. McGing, P., Reed, B., Weir, D.G. and Scott, J.M. (1978) Biochem. Biophys.Res.Commun. 82, 540-546.
7. Deacon, R., Lumb, M., Perry, J., Chanarin, I., Minty, B., Halsey, M.J. and Nunn, J.F. (1978) Lancet, ii, 1023-1024.
8. Killmann, S-A. (1964) Acta Med.Scand. 175, 483-488.
9. Metz, J., Kelly, A., Swett, V.C., Waxman, S. and Herbert, V. (1968) Br.J.Haematol. 14, 575-592.
10. Scott, J.M., Reed, B., McKenna, B., McGing, P., McCann, S., O'Sullivan, H., Wilson, P. and Weir, D.G. (1978) in Chemistry and Biology of Pteridines (Kisliuk, R.L. and Brown, G.M. ed.), pp 335-340. (Elsevier North-Holland, New York).
11. Perry, J., Chanarin, I., Deacon, R. and Lumb, M. (1979) Biochem. Biophys.Res.Commun. 91, 678-684.
12. Rabinowitz, J.C. (1963) Methods in Enzymology. (Colowick, S.P. and Kaplan, N.O. ed.), vol.6, pp. 814-815. (Academic Press, New York).
13. Ganeshaguru, K. and Hoffbrand, A.V. (1978) Br.J.Haematol. 40, 29-41.
14. Goodman, A., Pinchak, A. and Harris, J.W. (1979) Blood. 54, Suppl.1, 38a.
15. Lumb, M., Deacon, R., Perry, J., Chanarin, I., Minty, B., Halsey, M.J. and Nunn, J.F. (1980) Biochem. J. 186, in press.